PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: HOEKSTRA et al.

Serial No.: 09/385,918 Art Unit: 1646

Filed: August 30, 1999 Examiner: Andres, J.

For: METHODS FOR MODULATING SIGNAL TRANSDUCTION MEDIATED BY TGF-B AND RELATED PROTEINS

Attorney Docket No.: 10624-048

DECLARATION OF THE INVENTORS UNDER 37 C.F.R. § 1.131

Assistant Commissioner for Patents
Washington, DC 20231

We, MERL F. HOEKSTRA, WEILIN XIE, BRION W. MURRAY, and FRANK M. MERCURIO , do declare and state that:

1. We are the named inventors of the invention disclosed and claimed in the above-identified patent application, Serial No. 09/385,918.

2. Merl F. Hoekstra, who is no longer employed by Signal Pharmaceuticals, Inc., resides at 16322 224th Street SE, Monroe, WA 98272.

3. Xie Weilin is a senior scientist at Signal Pharmaceuticals, Inc. at 5555 Oberlin Drive, San Diego, CA 92121.

4. Brion W. Murray, who is no longer employed by Signal Pharmaceuticals, Inc., is employed by Agouron/Pfizer at 4215 Sorrento Valley Boulevard, San Diego, CA 92121-1408

a research scientist at Signal Pharmaceuticals, Inc. at 5555 Oberlin Drive, San Diego, CA 92121.

5. Frank M. Mercurio is Director, Cell Signaling and Target Discovery at Signal Pharmaceuticals, Inc. at 5555 Oberlin Drive, San Diego, CA 92121.

6. We conceived of and reduced to practice the invention described and claimed in the present application in the United States of America, prior to July 21, 1999.

7. Attached hereto as Exhibit 1 is a copy of a cover letter addressed to Ms. Sholita Packer of Signal Pharmaceuticals, Inc. from Ann T. Kadlecek at Seed and Berry LLP, 6300 Columbia Center, 701 Fifth Avenue, Seattle, Washington 98104-7092 and a first draft of the instant application that evidences our possession of the invention as claimed prior to July 21, 1999. Although the dates on the cover letter and draft of Exhibit 1 have been redacted, the letter and the draft application were received and reviewed by us prior to July 21, 1999.

8. This first draft application prepared and sent to Signal Pharmaceuticals by Ann T. Kadlecek in the United States discloses the subject matter of Zhu et al. (Nature, August 12, 1999, Vol. 400, pages 687-693) and Hustad et al. (United States Patent Number 6,087,122 filed July 21, 1999) relied upon by the Examiner in the Office Action mailed December 1, 2000 as detailed below.

8.1 The first draft application teaches HECT E3 ubiquitin ligases, which contain a WW domain, bind to a PY motif in certain Smad proteins (such as Smad 1 and Smad5) and result in ubiquitination and degradation of the target Smad.

8.2 The first draft application teaches E3 ubiquitin protein ligase can be used in screening assays.

8.3 The first draft application teaches the use of E3 ubiquitin protein ligase or variants thereof for screening candidate agents in assays in which the fragment is free or bound and the formation of binding complexes are tested.

9. We declare further that all statements made in this Declaration of our knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 1/09/02

Merl F. Hoekstra

Dated: 12/13/01

Xie Weilin

Dated: 1/18/2002

Brion W. Murray

Dated: 1/18/02

Frank M. Mercurio

Exhibit 1: A cover letter and first draft patent application prepared by Ann T. Kadlecek.

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VIA FEDERAL EXPRESS

Ms. Sholita Packer
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5555 Oberlin Drive
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Re: Draft Patent Application Entitled
METHODS FOR MODULATING BMP-MEDIATED SIGNALING
Our Reference: 860098.433
Contact Person: **Merl Hoekstra**

Dear Sholita:

Enclosed is the first draft of the above-identified application for your review and comments. Please pay particular attention to the questions in bold brackets. If you have any questions, please do not hesitate to call.

Very truly yours,

SEED and BERRY LLP

Ann T. Kadlecek

Ann T. Kadlecek

ATK:asl

Enclosure:
Application

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METHODS FOR MODULATING BMP-MEDIATED SIGNALING

TECHNICAL FIELD

The present invention relates generally to methods for identifying agents that modulate signaling mediated by bone morphogenic protein (BMP). The invention is more particularly related to screens for use in evaluating agents for the ability to modulate Smad protein degradation, and to methods using such agents to augment or inhibit BMP-mediated signaling in a variety of cell types.

BACKGROUND OF THE INVENTION

The transforming growth factor beta (TGF-β) superfamily is a large family of multifunctional proteins that regulate a variety of cellular functions, including cellular proliferation, migration, differentiation and apoptosis. One group of proteins within this superfamily is the family of bone morphogenic proteins (BMPs), which selectively transduce signals to responding cells through serine/threonine type transmembrane receptors. The signals are further transduced from the BMP receptors to the nuclei, resulting in altered patterns of gene expression. BMP-mediated signal transduction is important for a variety of normal processes, including bone growth and the function of the nervous system, eyes and organs such as kidneys **[EXPAND?]**.

Regulation of signal transduction from BMP receptors to nuclei remains incompletely understood. Signal transduction is known to involve Smad family proteins, certain of which become incorporated into transcriptional complexes and activate downstream genes. Smad proteins have been identified based on homology to the *Drosophila* gene Mothers against dpp (mad), which encodes an essential element in the *Drosophila* dpp signal transduction pathway (*see* Sekelsky et al., *Genetics 139*:1347-1358, 1995; Newfeld et al., *Development 122*:2099-2108, 1996). Smad proteins generally are characterized by highly conserved amino- and carboxy-terminal domains separated by a proline-rich linker **[EXPAND RE COMMON CHARACTERISTICS OF SMADS?]**.

To date, eight **[CORRECT?]** Smad proteins have been identified and shown to participate in signal responses induced by TGF-β family members (*see* Kretzschmar and Massague, *Current Opinion in Genetics and Development* 8:103-111, 1998). These Smads can be divided into three subgroups. One group (Smads1, 2, 3, 5 and 8) includes Smads that are direct substrates of a TGF-β family receptor kinase. Another group (Smad 4) includes Smads that are not direct receptor substrates, but participate in signaling by associating with receptor-activated Smads. The third group of Smads (Smad6 and Smad7) consists of proteins that inhibit activation of Smads in the first two groups.

Smads have specific roles in pathways of different TGF-β family members. In particular, following binding of a BMP to a BMP receptor, Smad1 and Smad5 are recruited to the receptor and phosphorylated. Once these proteins are phosphorylated, Smad1 and Smad5 form a complex with Smad4, and the complex translocates to the nucleus, resulting in activation of BMP-mediated gene transcription. The regulation of this process is poorly understood, and further knowledge of this signaling pathway is needed to facilitate the development of therapeutic agents that modulate BMP-mediated signaling.

Accordingly, there is a need in the art for an improved understanding of the molecular mechanisms of BMP-mediated signaling and for the development of agents that modulate such signaling. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides methods for identifying agents that modulate BMP-mediated signal transduction. Within certain aspects, such methods comprise the steps of (a) contacting (i) a first polypeptide comprising a HECT E3 ubiquitin ligase WW domain, or a variant thereof in which the ability of the polypeptide to bind to a Smad protein is not substantially diminished relative to the HECT E3 ubiquitin ligase; (ii) a second polypeptide comprising a Smad PY motif, or a variant thereof in which the ability of the polypeptide to bind to an E3 ubiquitin ligase

is not substantially diminished relative to a native Smad protein comprising the PY motif; and (iii) a candidate agent; wherein the step of contacting is performed under conditions that permit a detectable level of binding of the first polypeptide to the second polypeptide in the absence of candidate agent; (b) determining a level of binding of the first polypeptide to the second polypeptide; and (c) comparing the level of binding to a control level of binding of the first polypeptide to the second polypeptide in the absence of candidate agent, and therefrom determining whether the candidate agent modulates BMP-mediated signaling.

Within other aspects, such methods comprise the steps of: (a) contacting (i) a candidate agent; (ii) a ubiquitinated HECT E3 ubiquitin ligase; and (iii) a Smad protein or a variant thereof that comprises a PY motif; wherein the contact takes place under conditions and for a time sufficient to permit ubiquitination of the Smad protein or variant thereof by the HECT E3 ubiquitin ligase in the absence of candidate agent; (b) determining a level of ubiquitination of the Smad protein or variant thereof; and (c) comparing the level of ubiquitination to a control level of ubiquitination in the absence of candidate agent, and therefrom determining whether the candidate agent modulates BMP-mediated signaling.

Within further aspects, such methods comprise the steps of: (a) contacting a cell that expresses a BMP receptor with a bone morphogenic protein and a candidate agent; and (b) detecting a level of a Smad protein in the bone cell, relative to a level of the Smad protein in a cell that is contacted with the bone morphogenic protein in the absence of the candidate agent, and therefrom determining whether the candidate agent is a modulator of BMP-mediated signaling.

Still further such methods comprise the steps of: (a) contacting a cell that expresses a BMP receptor with a bone morphogenic protein and a candidate agent; and (b) detecting a level of ubiquitination of a Smad protein in the cell, relative to a level of the Smad protein ubiquitination in a cell that is contacted with the bone morphogenic protein but is not contacted with the candidate agent, and therefrom determining whether the candidate agent modulates BMP-mediated signaling.

Within other such aspects, a method for screening for an agent that modulates BMP-mediated signaling comprises the steps of: (a) contacting a cell that expresses a BMP receptor with bone morphogenic protein and a candidate agent; and (b) detecting a level of a HECT E3 ubiquitin ligase activity in the cell, relative to a level of HECT E3 ubiquitin ligase activity in a cell that is contacted with the bone morphogenic protein in the absence of the candidate agent, and therefrom determining whether the candidate agent modulates BMP-mediated signaling.

The present invention further provides, within other aspects, methods for augmenting BMP-mediated signaling in a cell, comprising contacting a cell with an agent that inhibits binding of a HECT E3 ubiquitin ligase WW domain to a Smad PY motif and/or inhibits ubiquitination of a Smad protein.

Within further aspects, the present invention provides methods for stimulating bone formation in a patient, comprising administering to a patient a therapeutically effective amount of an agent that inhibits binding of a HECT E3 ubiquitin ligase WW domain to a Smad PY motif and/or inhibits ubiquitination of a Smad protein.

The present invention further provides, within other aspects, methods for preventing or treating a condition associated with insufficient BMP-mediated cell signaling, comprising administering to a patient a therapeutically effective amount of an agent that inhibits binding of a HECT E3 ubiquitin ligase WW domain to a Smad PY motif and/or inhibits ubiquitination of a Smad protein.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram illustrating the general mechanism for ubiquitin (Ub) ligation to targeted proteins. Ubiquitination is initiated by ATP-dependent transfer of a ubiquitin monomer to enzyme 1 (E1) in the ubiquitin cascade. Following

ubiquitin activation on E1, a transfer of ubiquitin to a ubiquitin carrier protein (E2) occurs. Transfer of the ubiquitin to a targeted protein is mediated by ubiquitin ligases (E3's).

Figure 2 is a Western blot illustrating the induction of Smad1 degradation by BMP. The level of tagged Smad1 in transfected cells following treatment with BMP and/or LLF is shown, as indicated.

Figure 3 is a Western blot illustrating Smad1 ubiquitination. COS cells expressing HA-tagged Smad1 were treated with one or more of BMP, LLF and/or ubiquitin (Ub), as indicated. Cells were lysed and Smad1 was immunoprecipitated. Western blots were probed with Anti-HA and anti-ubiquitin antibodies, as indicated.

Figure 4 is an autoradiogram illustrating the binding of WWP1.1 to the PY motif of Smad1. Untransfected COS cells (COS), or COS cells transfected with HA-tagged Smad1 or altered Smad1 with a mutated PY motif (Smad1*), were lysed. Smad1 was immunoprecipitated and incubated with ^{32}P-labeled GST-fusion proteins of the WWP1.1 WW domain. Bound WWP1.1 was then detected autoradiographically, as indicated.

Figures 5A-5D are histograms illustrating the binding of PY motif peptides to WWP1 WW domain peptides. Four GST fusion peptides were assayed: GST-WWP1.1 (Figure 5A), GST-WWP1.2 (Figure 5B), GST-WWP1.3 (Figure 5C) and GST-WWP1.4 (Figure 5D). In each panel, binding to GST alone is also shown (cross-hatched columns, as indicated). WW domain peptides were coated on polystyrene plates at the indicated receptor coating concentrations, and blocked with BSA. Biotinylated PY motif peptides (Nedd, mutant Nedd and WBP1) were then added as indicated. Binding was assessed using a time-resolved fluorescence assay and is shown as binding activity (cps).

Figures 6A-6D are graphs illustrating the binding of Smad PY motif peptides to WWP1 WW domain peptides. Four GST fusion peptides were assayed, and are shown in each graph: GST-WWP1.1, GST-WWP1.2, GST-WWP1.3 and GST-WWP1.4. Binding to GST alone is also shown (open squares). WW domain peptides were coated on polystyrene plates at the indicated receptor coating concentrations, and

blocked with BSA. Biotinylated PY motif peptides (Smad7 (Figure 6A); Smad6 (Figure 6B); Smad2 (Figure 6C) and Smad3 (Figure 6D)) were then added as indicated. Binding was assessed using a time-resolved fluorescence assay and is shown as cps.

Figures 7A-7B are graphs illustrating the binding of Smad PY motif peptides to WWP1 WW domain peptides. Four GST fusion peptides were assayed, and are shown in each graph: GST-WWP1.1, GST-WWP1.2, GST-WWP1.3 and GST-WWP1.4. WW domain peptides were coated on polystyrene plates at the indicated receptor coating concentrations, and blocked with BSA. Biotinylated PY motif peptides (Smad5 (Figure 7A) and Smad1 (Figure 7B)) were then added as indicated. Binding was assessed using a time-resolved fluorescence assay and is shown as cps.

Figures 8A-8B are graphs illustrating the binding of increasing concentrations of a Smad7 PY motif peptide to WWP1 WW domain peptides. In Figure 8A, binding to four GST fusion peptides (GST-WWP1.1, GST-WWP1.2, GST-WWP1.3 and GST-WWP1.4) is shown, as well as binding to RSP5.2 WW domain. WW domain peptides were coated on polystyrene plates at the indicated receptor coating concentrations, and blocked with BSA. Biotinylated PY motif peptide was then added at the indicated concentrations. Binding was assessed using a time-resolved fluorescence assay and is shown as cps. Figure 8B presents a Scatchard analysis of the Smad7 PY motif to WWP1.2 and WWP1.4, as indicated.

Figures 9A-9C are autoradiograms illustrating the activation and activity of E1 in a coupled ubiquitination assay. Figure 9A shows ubiquitinated E1 (lane 2), where the presence of E1-covalently linked to labeled ubiquitin is shown by the indicated high molecular weight band. In figure 9B, bands indicating ubiquitinated E1 and E2 (UBC5c) are shown in lane 1, and this ubiquitination is not present in lane 2 (reaction performed in the absence of E1) or lane 3 (reaction performed in the presence of DTT). Figure 9C shows ubiquitinated E1 and E2 (UBC7) in lane 1, and this ubiquitination is not present in lane 2 (reaction performed in the absence of E1) or lane 3 (reaction performed in the presence of DTT).

Figures 10A-10C are autoradiograms illustrating the ubiquitination of the HECT E3 ligase WWP1 WW domain in a coupled ubiquitination assay. In each

Figure, incorporation of labeled ubiquitin into a WWP1 HECT domain containing residues 611-985 or 611-990 is shown, as indicated. Reactions were performed in the presence or absence of DTT, as indicated. Ubiquitinated WWP1-GST is indicated by the arrow. In Figure 10A the E2 was UBC5c, and in Figure 10B the E2 was UBS7. Controls (Figure 10C) were performed in the absence of E2.

Figures 11A-11C are autoradiograms illustrating the ubiquitination of the HECT E3 ligase WWP1 in a coupled ubiquitination assay. In each Figure, incorporation of labeled ubiquitin into a WWP1 HECT domain containing residues 611-985 is shown. Also shown are ubiquitinated E1 and E2. Reactions were performed in the presence or absence of DTT, as indicated. In Figure 11A the E2 was UBC5c, and in Figure 11B the E2 was UBS7. Controls (Figure 11C) were performed in the absence of E2 (lane 1) or in the absence of E1 and E2 (lane 2).

Figures 12A-12C are autoradiograms illustrating the time course of ubiquitination of the HECT E3 ligase WWP1 in a coupled ubiquitination assay. In each Figure, incorporation of labeled ubiquitin into a WWP1 HECT domain containing residues 611-985 following various incubation times, as indicated, is shown. In Figure 12A the E2 was UBC5c, and in Figure 12B the E2 was UBS7. A control (Figure 12C) was performed in the absence of E2, in a 60 minute reaction.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to methods for identifying agents that modulate BMP-mediated signaling, and to methods for using such agents for therapeutic purposes. The present invention is based, in part, on the discovery that BMP-mediated signaling is dampened by BMP-induced ubiquitin-mediated degradation of certain Smad proteins (such as Smad1 and Smad5). In particular, it has been found within the context of the present invention that HECT E3 ubiquitin ligases that contain a WW domain, bind to a PY motif in certain Smad proteins, resulting in ubiquitination and proteasome-mediated degradation of the target Smads. Agents that inhibit binding between a HECT E3 WW domain and a Smad PY

motif may generally be used to inhibit degradation of a Smad protein (*i.e.*, stabilize the Smad protein), resulting in enhanced BMP-mediated signaling.

Ubiquitin-mediated protein degradation is regulated by the ubiquitin conjugating pathway (Figure 1). Within this pathway, selective ubiquitination is initiated by ATP-dependent transfer of a ubiquitin monomer to enzyme 1 (E1) in the ubiquitin cascade. Ubiquitin bound to E1 is then activated with ATP to form an ubiquitin-AMP intermediate. The AMP is displaced by the E1 active site cysteine to form a thioester linkage with the carboxy terminus of ubiquitin. A second activated ubiquitin is then formed by E1, which allows the E1 to transfer ubiquitin from its active site cysteine to the active site cysteine of a ubiquitin carrier protein (E2). During this transfer, diversity in the ubiquitination pathway begins to initiate and amplify. The greatest degree of selectivity in the ubiquitination cascade occurs at the level of ubiquitin transfer, ligation and polymerization on selected substrates. This terminal step is mediated by ubiquitin ligases (E3's). E2 either transfers the ubiquitin from its active site to the cysteine of an E3 ubiquitin ligase or to the target protein in an E3-dependent manner. Following transfer and ligation of ubiquitin onto substrates by E3, the ubiquitinated protein is targeted for degradation by the 26S proteasome. Selectivity for proteasome-mediated protein degradation is determined by the ubiquitin tag.

As used herein, a HECT E3 ubiquitin ligase is an E3 ubiquitin ligase that contains a HECT (His-Glu-Cys-Thr; SEQ ID NO:__) sequence within the catalytic carboxyterminal domain. E3 ubiquitin ligases are members of the ubiquitination cascade that transfer ubiquitin to specific substrates, rendering the substrates targets for proteasome-mediated degradation. Known HECT E3 ubiquitin ligases include WWP1, E6-associated protein, Rsp5, Nedd4 _____ **[OTHERS? REFERENCES?])** and other HECT E3 ubiquitin ligases may be identified based on sequence similarity to known proteins and/or functional properties of HECT E3 ligases. A variety of techniques may be used to evaluate sequence similarity. One such technique is searches of sequence databases (*e.g.*, GenBank). Such searches may be performed using well known programs (*e.g.*, NCBI BLAST searches), and proteins that display high levels of sequence identity and/or similarity are candidate HECT E3 ligases. Alternatively,

techniques employing low stringency hybridization may facilitate the identification of a HECT E3 ligase. Within such techniques, a known HECT E3 ubiquitin ligase (or a portion thereof) is used as a probe to screen a library (cDNA or genomic) for hybridizing sequences. Suitable low stringency hybridization conditions include, but are not limited to, _____ **[PLEASE PROVIDE CONDITIONS]**. Yet another technique for evaluating sequence similarity employs PCR reactions that are performed using degenerate primers that encode a conserved sequence (such as His-Glu-Cys-Thr; SEQ ID NO:__).

Alternatively, a functional assay may be used to identify a HECT E3 ubiquitin ligase. Certain assays detect binding to substrates, such as Smad proteins or portions thereof (*e.g.*, a PY motif). Such assays are well known in the art, and include affinity purification, yeast two-hybrid screens and screens of phage display libraries. Methods for performing these and other binding assays are amply described in the patent and scientific literature (*e.g.*, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989; Brachman and Boeke, *Current Opinion in Biotechnology 8*:5661-568, 1997; and references cited therein). Other functional assays may be designed to assess ubiquitin transferring activity of a candidate HECT E3 ubiquitin ligase. Such assays may be performed using known techniques (*e.g.*, coupled ubiquitin assays or ubiquitin dependent proteolysis assays in which the activity of E3 for transferring ubiquitin to a substrate is coupled with a measurement of substrate proteolysis), which are described in greater detail below. A HECT E3 ubiquitin ligase should display detectable ubiquitin transferring activity within such assays.

A Smad protein is a protein that is homologous to a known Smad protein (*i.e.*, at least ____% identical **[HOW SIMILAR MUST A SEQUENCE BE?]**), and that participates in signal transduction mediated by a TGF-β family member (*i.e.*, expression of a Smad protein detectably enhances or inhibits such signal transduction as measured using any assay suitable for the particular TGF-β family member **[EXPAND ON DEFINITION OF SMAD PROTEINS?]**. Smad proteins of particular interest include Smad1 (**[REFERENCE]**), Smad2 (**[REFERENCE]**), Smad3

([REFERENCE]), Smad5 ([REFERENCE]), Smad6 ([REFERENCE]) and Smad7 ([REFERENCE]). It will be apparent, however, that any Smad protein that contains a PY motif as described herein may be stabilized using the methods provided herein.

ASSAYS FOR AGENTS THAT MODULATE BMP-MEDIATED SIGNALING

Screening assays for agents that modulate BMP-mediated signaling may be performed in a variety of formats, including cell-based and *in vitro* assays. In general, such an assay should evaluate the effect of an agent on: (1) binding of a HECT E3 ubiquitin ligase WW domain to Smad PY motif; (2) ubiquitination of a Smad protein by E3 ubiquitin ligase; (3) proteolysis of a Smad protein (*e.g.*, by assessing the cellular level of a Smad protein) or (4) HECT E3 ubiquitin ligase activity.

Candidate agents that may be screened within the assays provided herein include, but are not limited to, antibodies and antigen-binding fragments thereof, competing peptides that represent a WW domain or PY motif, and other natural or synthetic molecules, such as small molecule inhibitors, that bind to a HECT E3 ubiquitin ligase or Smad protein. Candidate agents may be present within a library (*i.e.*, a collection of compounds). Such agents may, for example, be encoded by DNA molecules within an expression library. Other such agents include compounds known in the art as "small molecules," which have molecular weights less than 10^5 daltons, preferably less than 10^4 daltons and still more preferably less than 10^3 daltons. Such candidate agents may be provided as members of a combinatorial library, which includes synthetic agents (*e.g.*, peptides) prepared according to multiple predetermined chemical reactions. Those having ordinary skill in the art will appreciate that a diverse assortment of such libraries may be prepared according to established procedures, and members of a library of candidate agents can be simulatneously or sequentially screened as described herein.

In vitro assays may be used for rapid screening of candidate agents for the ability to inhibit binding of a HECT E3 ubiquitin ligase to a Smad protein. As noted above, this binding has been found, within the context of the present invention, to take place between the WW domain of HECT E3 ubiquitin ligase and the PY motif of

certain Smad proteins. Accordingly, any *in vitro* assay that assesses the effect of a candidate agent on this interaction may be used to identify agents that modulate BMP-mediated signaling. Such assays typically assess the effect of an agent on binding between a polypeptide comprising a HECT E3 WW domain, or a variant thereof, and a polypeptide comprising a Smad PY motif, or a variant thereof.

A HECT E3 WW domain, as used herein, is a region of a HECT E3 ubiquitin ligase that contains two tryptophan residues 20 to 22 amino acid residues apart (*see* M. Sudol, *Prog. Biophys. Molec. Biol. 65*:113-132, 1996), and detectably binds to a Smad PY motif, as described herein. Within preferred embodiments, a WW domain satisfies the following consensus sequence **[CAN WE GENERATE ONE FOR THE HECT E3s?]**:

Representative HECT E3 ubiquitin ligase WW domains include **[CAN WE SHORTEN THESE?]**: SPLPPGWEERQDILGRTYYVNHESRRTQWKRPTPQDNL (human Nedd4; SEQ ID NO:__), SGLPPGWEERQDILGRTYYVNHESRRTQWKRPTPQDNL (human Nedd4; SEQ ID NO:__), GFLPKGWEVRHAPNGRPFFIDHNTKTTTWEDPRLKIPA (human Nedd4; SEQ ID NO:__), GPLPPGWEERTHTDGRIFYINHNIKRTQWEDPRLENVA (human Nedd4; SEQ ID NO:__), GRLPPGWERRTDNFGRTYYVDHNTRTTTWKRPTLDQTE (yeast Rsp5; SEQ ID NO:__); GELPSGWEQRFTPEGRAYFVDHNTRTTTWVDPRRQQYI (yeast Rsp5; SEQ ID NO:__); GPLPSGWEMRLTNTARVYFVDHNTKTTTWDDPRLPSSL (yeast Rsp5; SEQ ID NO:__); _____ **[PLEASE PROVIDE OTHER KNOWN WW DOMAIN SEQUENCES FOR HECT E3 UBIQUITIN LIGASES - ESPECIALLY THE WWP1 WW DOMAINS?]**. Within the assays provided herein, a polypeptide comprising a WW domain may be a full length HECT E3 ubiquitin ligase, a portion thereof that comprises a WW domain, or a variant of such a polypeptide in which the WW domain is modified by one or more substitutions, additions, insertions and/or deletions such that the ability of the variant to bind to a Smad PY motif is not substantially diminished (*i.e.*, is enhanced, unchanged or diminished by no more than

10%), relative to the native WW domain sequence. This binding activity may be assessed using a representative binding assay provided herein.

A Smad PY motif is a region **[HOW LONG?]** of a Smad protein that contains a PPxY (Pro-Pro-Xaa-Tyr; SEQ ID NO:__) sequence, in which x and Xaa both represent any amino acid. Such a PY motif further binds detectably to a HECT E3 ubiquitin ligase WW domain, as provided herein. Representative Smad PY motifs are present, for example, within Smads 1, 2, 3, 5, 6 and 7. Smad PY motifs preferably satisfy the consensus sequence Ser/Thr-Pro-Pro-Pro-Pro/Ala/Gly-Tyr (SEQ ID NO:__), wherein Ser/Thr is an amino acid residue that is serine or threonine and Pro/Ala/Gly is an amino acid residue that is selected from the group consisting of proline, alanine and glycine **[IS THIS A REASONABLE CONSENSUS SEQUENCE?]**. Polypeptides comprising a Smad PY motif may comprise, for example, a sequence such as ELESPPPPYSRYPM (SEQ ID NO:__), GPESPPPPYSRLSP (SEQ ID NO:__), PADTPPPAYLPPED (SEQ ID NO:__), PADTPPPAYMPPDD (SEQ ID NO:__), IPETPPPGYISEDG (SEQ ID NO:__) or AGLTPPPGYLSEDG (SEQ ID NO:__). Within the assays provided herein, a polypeptide comprising a PY motif may be a full length Smad protein, a portion thereof that comprises a PY motif, or a variant of such a polypeptide in which the PY motif is modified by one or more substitutions, additions, insertions and/or deletions such that the ability of the variant to bind to a HECT E3 ubiquitin ligase WW domain is not substantially diminished (*i.e.*, is enhanced, unchanged or diminished by no more than 10%), relative to the native PY motif sequence. This binding activity may be assessed using a representative binding assay provided herein.

Preferably, a WW domain or PY motif polypeptide variant contains conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For

example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the secondary structure and hydropathic nature of the polypeptide.

WW domain and PY motif polypeptides may comprise additional sequences that are unrelated to an endogenous protein. Such sequences include signal (or leader) sequences at the N-terminal end of the protein that co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.*, poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

The WW domain polypeptide and PY motif polypeptide are contacted under conditions that permit binding between the two polypeptides in the absence of a candidate agent. Candidate agent may be added to the reaction mixture before or after contact of the WW domain polypeptide with the PY motif polypeptide. The reaction is then incubated, and binding of the WW domain polypeptide to the PY motif polypeptide is assessed, using any standard technique. One suitable binding assay employs a solid support, as described above, to which one of the polypeptides is attached. Binding may be assessed by removing unbound substances and detecting the presence of the other polypeptide on the solid support. Such detection may be achieved using, for example, an antibody or antigen-binding fragment detection reagent, or using a competitive assay with labeled polypeptide, as described above. Alternatively, the

polypeptide that is not immobilized on the support may itself comprise a tag that facilitates detection of bound polypeptide. Tags include, but are not limited to, biotin, enzymes, radioactive groups (*e.g.*, ^{32}P), dyes, luminescent groups, fluorescent groups and other sequences that are readily bound by a detection reagent (*e.g.*, antigenic sequences specifically bound by particular antibodies). In general, an agent should detectably modulate binding between the WW domain polypeptide and PY motif polypeptide.

By way of example, one polypeptide (*i.e.*, a WW domain polypeptide or PY motif polypeptide) may be immobilized through non-specific interactions (*e.g.*, to a polystyrene plate) or through a protein tag interaction (*e.g.*, an interaction between a His_6-fusion protein and a nickel plate). The polypeptide may be immobilized by, for example, contacting a polystyrene assay plate (Costar) with the polypeptide overnight at 4°C in a 200 mM carbonate buffer (Pierce, Rockford IL) at a concentration ranging from 0.3 to 30 μg/mL. Unbound polypeptide may be removed by washing with distilled, deionized water and the plates may then be blocked with 1% BSA/carbonate buffer for two hours at room temperature. Plates may then be washed with Tris/Tween buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% BSA, 1 mM DTT 0.1% Tween20, protease inhibitor cocktail (Boehringer-Mannheim). The other polypeptide may be labeled (*e.g.*, biotinylated) and allowed to bind to the immobilized polypeptide (*e.g.*, solvated in Tris/Tween buffer and incubated in the assay plates at 4°C for varying amounts of time). Plates may then be washed with PBS/0.1% Triton X100. Binding may be detected by, for example, probing the assay wells with 1 μg/mL Europium-labeled streptavidin (DELFIA; Wallac Oy, Turku, Finland) in DELFIA Assay Buffer/0.1% Triton X100 for one hour at room temperature. Unbound Europium-labeled streptavidin may be removed by washing with PBS/0.1% Triton X100. Europium may be released for time-resolved fluorescence (TRF) measurements with the DELFIA Enhancement Buffer. TRF measurements may be made, for example, with the DELFIA 1234 (Wallac Oy, Turku, Finland) or Victor ([CITY? STATE?]) fluorometer.

Within similar assays, a radioactive label may be substituted for the biotin. For example, a ^{32}P-labeled polypeptide may be generated by phosphorylation of a suitable site linked to the WW domain polypeptide or PY motif polypeptide. One such site is the PKA site in the pGEX KG vector (Pharmingen), which may be labeled using α-[^{32}P]-ATP and protein kinase A (Sigma). The amount of binding may be quantitated by, for example, Cerenkov counting or SDS-PAGE using standard techniques. The solid support used may also be varied. One suitable support for such assays is neutravidin agarose beads (Pierce, Rockford, IL). Binding may be performed using such a support by incubation in a PBS/1% Tween20 buffer in an end-over shaker at 4°C for varying amounts of time. It will be apparent that any of these assays may be modified to permit immobilization after binding takes place.

To determine the effect of a candidate agent on binding of the WW domain to the PY motif, the level of binding is compared in the presence and absence of candidate agent. An agent that detectably inhibits or enhances such binding may be used to alter BMP-mediated signaling in a cell.

Other *in vitro* assays may be designed to assess the effect of an agent on ubiquitination of an E3 ubiquitin ligase and/or a Smad. *In vitro* ubiquitination reactions are well known in the art. For example, coupled ubiquitination assays (in which ubiquitin transfer from E1 to E2, and from E2 to E3, is monitored) may be employed. Such assays require the reconstitution of an E1/E2/E3 pathway. Recombinant E1 and E2 components are available from a variety of sources (*e.g.*, BostonBiochem, Cambridge, MA) for coupling ubiquitin to an E3 ligase of interest. Radiolabeled ubiquitin may be generated using standard techniques, such as PKA-mediated incorporation of [^{32}P]-phosphate from α-[^{32}P]-ATP to the PKA site of the GST-ubiquitin fusion protein (pGEX KG expression vector). One suitable ubiquitin assay buffer is: 50 mM Tris pH7.6, 1 mM ATP, 0.2 mM EDTA, 5 mM $MgCl_2$, 1 unit inorganic pyrophosphatase, 0.005% Triton X100 and 1 µM staurosporine. In a 0.030 mL reaction, the following amounts of reaction components are generally suitable: 50-200 ng E1, 0.1-1µg E2, 5 µg GST-ubiquitin (BostonBiochem, Cambridge, MA) and _____ **[HOW MUCH?]** E3. Reactions may be performed at room temperature and

terminated with a SDS-PAGE loading buffer that does not contain mercaptans. Reactions may be analyzed by SDS-PAGE. An assay may be similarly performed with endogenous proteins from, for example, HeLa cell extract fractions (*see* Hershko et al., *J. Biol. Chem. 258*:8206-8214, 1983). For measuring Smad protein ubiquitination, a Smad polypeptide is included in the reaction. These assays may be further modified to measure Smad protein degradation by incorporation of the 20S proteasome into the assay **[SOURCE? HOW MUCH?]**.

A Smad polypeptide for use within such an assay may be tagged to facilitate detection of covalently attached ubiquitin. Such a polypeptide may be a full length Smad protein, or may be a truncated protein or a variant thereof, provided that the polypeptide contains a functional PY motif and ubiquitination site **[EXPAND?]**. Similarly, a HECT E3 ubiquitin ligase for use within such assays may be a full length protein, a truncated protein or a variant thereof, provided that the ligase contains a functional WW domain and HECT domain and ubiquitinates a Smad protein of interest.

Cell-based assays (*i.e.*, assays in which intact cells are exposed to a candidate agent) may be used to detect the effect of an agent on Smad protein degradation in a cellular environment. Such assays may be performed using any cell that expresses a bone morphogenic protein (BMP) receptor. Known BMP receptors include _____ **[SPECIFIC ONES OF PARTICULAR INTEREST? REFERENCES?]**. Suitable cells may be readily identified using immunochemical methods (employing antibodies raised against known BMP receptors), by direct measurement of BMP binding to the cells or by the detection of a BMP-mediated response in the cells following exposure to BMP. Such methods are well known in the art **[EXPAND? WHAT IS THE BEST WAY TO DETERMINE WHETHER A CELL EXPRESSES A BMP RECEPTOR?]**. In general, a cell should express a level of BMP receptor that is detectable using any such assay. For certain methods, a relatively high level of BMP receptor expression may be preferred **[CRITERIA FOR OPTIMAL BMP RECEPTOR EXPRESSION?]**. Cells that express a BMP receptor include, but are not limited to, bone cells, neurons **[CORRECT?]**, kidney cells, _____ **[OTHERS?]**.

A cell that expresses a BMP receptor is contacted with an amount of a BMP that is sufficient to result in a detectable level of BMP-mediated signaling in the cell, using any assay for BMP-mediated gene expression that is appropriate for the particular cell type. It will be apparent that the selection of a particular BMP will depend on the cell type. Such assays may be based on the detection of enhanced expression of BMP-regulated genes (*e.g.*, _____ **[LIST SOME?]**). Any standard assay, such as a hybridization or amplification-based assay, or an assay for expression of a reporter gene operably linked to a BMP-regulated promoter, may be used to detect enhanced expression of BMP-regulated genes. Alternatively, such as assay may be a functional assay. For example, BMP stimulates differentiation of osteoblasts **[BEST ASSAY?]**. Contact of such cells with BMP should be sufficient to result in differentiation, as detected by _____ **[HOW?]**. In general, contact of a cell with _____ **[HOW MUCH?]** BMP for _____ **[HOW LONG?]** is sufficient to result in a detectable level of BMP-mediated signaling in the cell.

To determine the effect of a candidate agent on BMP-mediated signaling, a cell is contacted with BMP as described above and with a candidate agent. A cell may be contacted with both substances simultaneously or sequentially, in either order. The amount of agent employed will vary, depending on the type of agent and the specific assay used, but in general ____ to ____ **[BROAD RANGE?]** of a candidate agent is sufficient.

Following contact with BMP and the candidate agent, BMP-induced Smad protein degradation (preferably Smad1, 2, 3, 5, 6 or 7 degradation), is assessed. It will be apparent that any of a variety of assays may be used to assess Smad protein degradation including, but not limited to, assays that detect the level of: (1) a Smad protein; (2) ubiquitination of a Smad protein; or (3) HECT E3 ubiquitin ligase activity in the cell. **[OTHER POSSIBLE CELL-BASED ASSAYS?]** In each type of assay (described in greater detail below), the level detected is compared with a level detected in the same type of cell, under the same conditions, but in the absence of candidate agent. A statistically significant difference in the signal detected in the presence of

candidate agent, relative to the signal detected in the absence of candidate agent, indicates that the agent modulates BMP-mediated signaling in the cell.

To assess the level of a Smad protein, well known immunochemical methods may be employed. Such methods typically use an agent, such as an antibody or antigen-binding fragment thereof, that specifically binds to the Smad protein. To perform such assays, cells are generally lysed and the lysate (with or without pretreatment) is contacted with antibody under conditions that permit antigen-specific binding. Bound antibody is then detected by means of a suitable detection reagent.

There are a variety of assay formats that may be used to detect the level of a Smad protein in a cell lysate. *See*, *e.g.*, Harlow and Lane, *Antibodies: A Laboratory Mamual*, Cold Spring Harbor Laboratory, 1988. In one embodiment, an assay involves the use of binding agent immobilized on a solid support to bind to and remove the Smad protein from the remainder of the lysate. The bound Smad protein may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/Smad complex. Such detection reagents may comprise, for example, an antibody that specifically binds to the Smad protein. Alternatively, a competitive assay may be used, in which a Smad protein or portion thereof is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the lysate. The extent to which components of the lysate inhibit the binding of the labeled Smad polypeptideto the binding agent is indicative of the level of the Smad protein in the lysate.

A solid support for use in such assays may be any material known to those of ordinary skill in the art to which a binding agent may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the

binding agent and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time (typically between about 1 hour and about 1 day). In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10 μg, and preferably about 100 ng to about 1 μg, is sufficient to immobilize an adequate amount of binding agent.

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the lysate, such that a Smad protein within the sample is allowed to bind to the immobilized antibody (*e.g.*, incubation for 30 minutes at room temperature). Unbound sample is then removed from the immobilized Smad-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the Smad protein) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

Alternatively, functional assays may be used to determine the level of a Smad protein. **[TRUE? IF SO, PLEASE DESCRIBE SUITABLE ASSAYS]**

The level of ubiquitination of a Smad protein may be readily determined based on the alteration in electrophoretic mobility of the ubiquitinated protein. Briefly, cells may be lysed and proteins present within the lysate may be separated by SDS-PAGE. A protein of interest may be detected by Western blot analysis. Ubiquitination

results in a shift in the apparent molecular weight of the protein to the higher molecular weight region of the gel. Quantitative or semi-quantitative results may be obtained using labeled secondary antibodies, or other detection reagents known in the art.

HECT E3 ubiquitin ligase activity in the cell may be evaluated by any of a variety of ubiquitination assays commonly used in the art. Such assays typically employ a tagged target protein and/or labeled ubiquitin. Ligase activity is then assessed using, for example, a coupled ubiquitination assay as described herein. Such assays generally employ E3 ubiquitin ligase (generally within a lysate, or partially or substantially purified from a cell lysate) to ubiquitinate the tagged target protein. Using radiolabeled ubiquitin, for example, the amount of ubiquitination of target protein may be determined by scintillation counting following removal of unbound ubiquitin. Alternatively, the degradation of target protein may be directly assessed by SDS-PAGE resolution of the reactions and detection of the tag. Assays to detect ubiquitination and degradation of proteins are well known in the art, and representative assays are described herein.

In general, the effect of an agent on BMP-mediated signaling may be determined based on its activity within the above assays. For Smads that enhance BMP-mediated signaling (including Smads 1 and 5), agents that inhibit Smad protein degradation may be used to augment BMP-mediated signaling. Similarly, agents that enhance degradation of such Smad proteins may be used to inhibit BMP-mediated signaling. Agents identified using the screens provided herein may be used within a variety of therapeutic contexts, as described in further detail below.

METHODS OF USE FOR AGENTS THAT MODULATE BMP-MEDIATED SIGNALING

Agents that modulate BMP-mediated signaling may be used for the prevention or treatment of conditions associated with insufficient or excess BMP-mediated signaling in certain cell types. In general, an agent that augments BMP-mediated signaling (*e.g.*, inhibits binding of a HECT E3 ubiquitin ligase WW domain to a Smad1 or Smad5 PY motif) is useful for stimulating bone anabolism, _____ **[PLEASE LIST OTHER USES]**. Agents that inhibit BMP-mediated

signaling may be used, for example, within therapies for cancer, inflammation, aging and infectious diseases **[ARE THESE CORRECT? OTHER USES?]**.

For administration to a patient, one or more agents are generally formulated as a pharmaceutical composition, which may be a sterile aqueous or non-aqueous solution, suspension or emulsion, and which additionally comprises a physiologically acceptable carrier (*i.e.*, a non-toxic material that does not interfere with the activity of the active ingredient). Any suitable carrier known to those of ordinary skill in the art may be employed in a pharmaceutical composition. Representative carriers include physiological saline solutions, gelatin, water, alcohols, natural or synthetic oils, saccharide solutions, glycols, injectable organic esters such as ethyl oleate or a combination of such materials. Such compositions may also comprise buffers (*e.g.*, neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.*, glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, antimicrobial compounds, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), inert gases and/or preservatives. Compositions of the present invention may also be formulated as a lyophilizate. Pharmaceutical compositions may also contain other compounds, which may be biologically active or inactive.

The compositions described herein may be administered as part of a sustained release formulation (*i.e.*, a formulation such as a capsule that effects a slow release of compound following administration). Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain a polypeptide, polynucleotide or modulating agent dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of release. The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Another delivery system for such agents is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (*i.e.*, an artificial membrane vesicle). It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticuloendothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

To achieve cell or tissue specificity, agents may (in some instances) be administered topically. Other agents may be specific for a particular HECT E3/Smad protein interaction, and thus may have a specific target cell type or tissue. It may, however, be beneficial in certain instances to employ a targeting moiety to facilitate delivery of an agent to a desired site. A targeting moiety is any compound (*e.g.*, a monoclonal or polyclonal antibody, a protein or a liposome) or cell that facilitates the delivery of the agent to a target cell or tissue, thereby increasing the local concentration of the agent. Targeting moieties include antibodies or fragments thereof, receptors, ligands and other molecules that bind to cells of, or in the vicinity of, the target tissue. An antibody targeting agent may be an intact (whole) molecule, a fragment thereof, or a functional equivalent thereof. Examples of antibody fragments are F(ab')2, -Fab', Fab and F[v] fragments, which may be produced by conventional methods or by genetic or protein engineering. Linkage is generally covalent and may be achieved by, for

example, direct condensation or other reactions, or by way of bi- or multi-functional linkers. Targeting moieties may be selected based on the cell(s) or tissue(s) at which the agent is expected to exert a therapeutic benefit.

As noted above, patients that may benefit from treatment with an agent that modulates BMP-mediated signaling are those that are afflicted with (or at risk for developing) a condition associated with insufficient or excess BMP-mediated signaling in certain cell types. Such conditions may be diagnosed using criteria accepted in the art for the condition. For example, _____ **[EXPAND RE SOME CONDITIONS?]**.

Agents may be administered to a patient by any procedure that is appropriate for the condition to be treated including, for example, topical, oral, nasal, intrathecal, rectal, vaginal, sublingual or parenteral administration, such as subcutaneous, intravenous, intramuscular, intrasternal, intracavernous, intrameatal or intraurethral injection or infusion. An appropriate dosage and a suitable duration and frequency of administration will be determined by such factors as the condition of the patient, the type and severity of the patient's disease, the particular form of the active ingredient and the method of administration. In general, an appropriate dosage and treatment regimen provides the agent(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit (*e.g.*, an improved clinical outcome, such as more frequent complete or partial remissions, or longer disease-free and/or overall survival). For prophylactic use, a dose should be sufficient to prevent, delay the onset of or diminish the severity of a condition associated with BMP-mediated signaling. Optimal dosages may generally be determined using experimental models and/or clinical trials. The use of the minimum dosage that is sufficient to provide effective therapy is usually preferred. Patients may generally be monitored for therapeutic or prophylactic effectiveness using assays suitable for the condition being treated or prevented, which will be familiar to those of ordinary skill in the art. Suitable dose sizes will vary with the size of the patient, but will typically range from about 10 mL to about 500 mL for 10-60 kg animal.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

Ubiquitination of Smad Proteins and Initial Characterization of E3/Smad Protein Binding

This Example illustrates the BMP-induced ubiquitination of Smad proteins, and the identification of a HECT E3 ligase domain that binds to Smad proteins.

HA-tagged Smad1 expression vector was transfected into COS cells. The transfected cells were then treated with BMP **[HOW MUCH?]** for 4 hours in the absence or presence of the proteasome inhibitor Leu-Leu-Phe (LLF) **[HOW MUCH?]**. The cells were lysed and equal amount of protein was loaded in each lane. Western analysis was perfomed using anti-HA antibody **[SOURCE?]**. As shown in Figure 2, BMP induces Smad1 degradation and LLF blocks BMP-induced Smad1 degradation.

To assess the *in vivo* ubiquitination of Smad1, a Smad1 expression vector was transfected into COS cells. The transfected cells were treated with BMP and the proteasome inhibitor LLF, as described above. The cells were then lysed and equal amounts of protein were used for immunoprecipitation using anti-HA antibody to precipitate tagged-Smad1 protein. The immunoprecipitated Smad1 was run on SDS-PAGE, followed by a Western blot using anti-HA antibody or anti-ubiquitin antibody (Babco). Cells treated with LLF and cells treated with LLF plus BMP clearly accumulated more high molecular weight, ubiquinated-Smad1 protein (Figure 3).

To analyze the *in vitro* binding of Smads with various WW domains, HA-tagged Smad1 was expressed in COS cells. The expressed Smad1 was immunoprecipitated from the cell lysate. After extensive washing, the immunoprecipitated Smad1 was mixed with ^{32}P-lableled GST-fusion proteins of WWP1 WW domains. The binding products were washed and run on SDS-PAGE. COS extract and ^{32}P-labeled GST protein was used as controls. WWP1.1 **[PLEASE PROVIDE SEQUENCE]** was found to bind to Smad1 (Figure 4, lane 2), but not the

Smad1 mutated at the PY motif (Figure 4, lane 3) **[PLEASE PROVIDE SEQUENCE OF MUTATED PY MOTIF]**.

These results indicate that BMP induces ubiquitination of Smad proteins, using a pathway that includes a HECT E3 ubiquitin ligase.

Example 2

Interaction Between HECT E3 Ubiquitin Ligase WW Domain and Smad Proteins PY Motif Peptides

This Example illustrates the binding of HECT E3 WW domains to PY motifs.

The HECT domain E3 ligase WWP1 has 4 WW domains (WWP1.1, 1.2, 1.3, 1.4) which interact with the WBP-1 PY motif peptide **[REFERENCE? PLEASE PROVIDE SEQUENCE FOR EACH DOMAIN]**. Each domain was individually expressed as a GST fusion protein. A TRF binding assay was used to evaluate interactions of PY motif peptides with these domains. WW domains were bound to a 96-well polystyrene assay plate (Costar) overnight at 4°C in a 200 mM carbonate buffer (Pierce, Rockford, IL) at different concentrations (0, 1, 3, 10 µg/mL). Four WW domains were used: GST-WWP1.1, GST-WWP1.2, GST-WWP1.3, GST-WWP1.4, and GST. Unbound WW domain was washed away with distilled, deionized water and the plates were blocked with 1% BSA/carbonate buffer for 2 hours at room temperature. The plates were then washed with Tris/Tween buffer: 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% BSA, 1 mM DTT, 0.1% Tween20, protease inhibitor cocktail (Boehringer-Mannheim).

PY motif peptides were synthesized with a C_6-linker and biotin tag. The following PY motifs were used **[PLEASE PROVIDE SEQUENCES]**:

WBP1

Nedd

Mutant Nedd

These biotinylated peptides were solvated in Tris/Tween buffer and added to the assay plates (30 μM). The plates were incubated at 4°C for varying amounts of capture time. The plates were then washed with PBS/0.1% Triton X100 and probed for 1 hour at room temperature with 1 μg/mL Europium-labeled streptavidin (DELFIA; Wallac Oy, Turku, Finland) in DELFIA Assay Buffer/0.1% Triton X100. The unbound Europium-labeled streptavidin was washed with PBS/0.1% Triton X100. Europium was released for time-resolved fluorescence measurements with the DELFIA Enhancement Buffer. Measurements were made on either the DELFIA 1234 or Victor fluorometers.

The WBP1 peptide bound specifically to the WWP1 WW domains but not to GST (Figures 5A-5D). The other biotinylated peptides did not specifically interact with either the WW domains or GST (Figures 5A-5D).

Smad PY motif peptides from Smad 1, 2, 3, 5, 6 and 7 (Table I) were then evaluated with the WW domains from WWP1 (Figures 6A-6D and 7A-7B). The GST-WW domain fusion proteins and GST alone were coated at 30 μg/mL overnight. After blocking the wells with BSA, the WW domain peptides were titrated with the Smad PY motif peptides. Smad 7 peptide demonstrated a very potent interaction with the second WW domain of WWP1 (WWP1.2) (Figures 6A-6D); much more potent than the reported WBP1 PY peptide (Figures 5A-5D). The Smad 5 and Smad 6 peptides had measurable interactions with the WWP1 WW domains but were modest compared to the Smad 7 interactions (Figures 6A-6D and 7A-7B). There was no measurable interaction of PY motif peptides from Smad 1, 2 or 3.

Table I

Smad Protein PY Motifs

Smad Protein	PY Motif Peptide
Smad7	ELESPPPPYSRYPM (SEQ ID NO:__)
Smad6	GPESPPPPYSRLSP (SEQ ID NO:__)

Smad1	PADTPPPAYLPPED (SEQ ID NO:__)
Smad5	PADTPPPAYMPPDD (SEQ ID NO:__)
Smad2	IPETPPPGYISEDG (SEQ ID NO:__)
Smad3	AGLTPPPGYLSEDG (SEQ ID NO:__)

A detailed evaluation of the interactions of Smad 7 with the WWP1 WW domains and the second WW domain from RSP5 was undertaken. GST was used to correct for nonspecific background interactions. The peptide titration of the WW domains was evaluated by a nonlinear, least squares fit of the data and Scatchard analysis (Figures 8A-8B). Both methods showed that WWP1.2 had very specific interactions with Smad 7 peptide (K_d = 2.4 μM). Binding interactions from WWP1.1 and WWP1.3 did not yield a linear plot in the Scatchard analysis.

These results indicate that HECT E3 ubiquitin ligase WW domains bind to Smad protein PY motifs.

Example 3

Coupled Ubiquitin Assay for Detecting HECT E3 Ligase Ubiquitination

This Example illustrates a coupled enzymatic assay that evaluates the fate of a labeled ubiquitin molecule in the E1/E2/E3 pathway.

Recombinant E1 (ubc5c) and E2 (ubc7) components were obtained from BostonBiochem (Cambridge, MA). Radiolabeled ubiquitin was generated by PKA-mediated incorporation of [^{32}P]-phosphate from α-[^{32}P]-ATP to the PKA site of the GST-Ub fusion protein (pGEX KG expression vector). The ubiquitin assay buffer (UbB) was as follows: 50 mM Tris pH7.6, 1 mM ATP, 0.2 mM EDTA, 5 mM $MgCl_2$, 1 unit inorganic pyrophosphatase, 0.005% Triton X100, and 1 μM staurosporine. In a 0.030 mL reaction, the following components were present: 50-200 ng E1, 0.1-1 μg E2 and 5 μg GST-Ub. Reactions were run at room temperature and terminated with a SDS-PAGE loading buffer that did not contain mercaptans. Reactions were analyzed

by SDS-PAGE. The ubiquitination of active site cysteine residues of E1 and E2s (ubc 5c and ubc 7) was observed (Figures 9A-9C). The addition of 20 mM DTT prevented the formation of the thioester intermediates (Figures 9B-9C).

To evaluate transfer to a HECT E3 ligase, assays were performed as above, with the addition of ____ **[HOW MUCH?]** of the WWP1 HECT domain containing residues 611-985 or 611-990 of WWP1, as indicated **[PLEASE PROVIDE SEQUENCE]**. The HECT domain of WWP1 was shown to be charged by either E1/ubc5C or E1/ubc7 (Figures 10A-10C and 11A-11C). The shorter HECT domain, WWP1(611-985), only became charged with one ubiquitin molecule, presumably on its active site cysteine. The sensitivity of the ubiquitin adduct with WWP1(611-985) to DTT is consistent with the bond being to the active site cysteine (Figures 11A and 11B, compare lane 4 to lane 2). The longer HECT domain, WWP1(611-990), displayed a lack of substrate selectivity (Figures 10A and 10B). Time courses of the WWP1(611-990) reactions mediated by either ubc5c or ubc7 indicated that the E2 ubc5c was more efficient at activating WWP1(611-990) (Figures 12A-12C). The loss of GST-ubiquitin correlated with the appearance of high molecular species (>201 kDa).

These results confirm the role of HECT E3 ubiquitin ligases in Smad protein degradation.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the present invention is not limited except by the appended claims.

Claims

1. A method for screening for an agent that modulates BMP-mediated signaling, comprising the steps of:

(a) contacting

(i) a first polypeptide comprising a HECT E3 ubiquitin ligase WW domain, or a variant thereof in which the ability of the polypeptide to bind to a Smad protein is not substantially diminished relative to the HECT E3 ubiquitin ligase;

(ii) a second polypeptide comprising a Smad PY motif, or a variant thereof in which the ability of the polypeptide to bind to an E3 ubiquitin ligase is not substantially diminished relative to a native Smad protein comprising the PY motif; and

(iii) a candidate agent; under conditions that permit a detectable level of binding of the first polypeptide to the second polypeptide in the absence of candidate agent;

(b) determining a level of binding of the first polypeptide to the second polypeptide; and

(c) comparing the level of binding to a control level of binding of the first polypeptide to the second polypeptide in the absence of candidate agent, and therefrom determining whether the candidate agent modulates BMP-mediated signaling.

2. A method according to claim 1, wherein the HECT E3 ubiquitin ligase WW domain comprises the sequence _____ **[CONCENSUS WW DOMAIN SEQUENCE FOR HECT E3 LIGASES?]**.

3. A method according to claim 1, wherein the Smad PY motif comprises the sequence Ser/Thr-Pro-Pro-Pro-Pro/Ala/Gly-Tyr (SEQ ID NO:__), wherein Ser/Thr is an amino acid residue that is serine or threonine and Pro/Ala/Gly is an amino acid residue that is selected from the group consisting of proline, alanine and glycine.

4. A method according to claim 1, wherein the candidate agent is a small molecule within a combinatorial library.

5. A method according to claim 1, wherein the first polypeptide is immobilized on a solid support and the second polypeptide comprises a tag.

6. A method according to claim 1, wherein the second polypeptide is immobilized on a solid support and the first polypeptide comprises a tag.

7. A method according to claim 5 or claim 6, wherein the tag is biotin or a radioactive group.

8. A method according to claim 1, wherein the level of binding is determined via a two-antibody sandwich assay.

9. A method according to claim 1, wherein the level of binding is determined via a competitive assay.

10. A method for screening for an agent that modulates BMP-mediated signaling, comprising the steps of:

(a) contacting

(i) a candidate agent;

(ii) a ubiquitinated HECT E3 ubiquitin ligase; and

(iii) a Smad protein or a variant thereof that comprises a PY motif;

wherein the contact takes place under conditions and for a time sufficient to permit ubiquitination of the Smad protein or variant thereof by the HECT E3 ubiquitin ligase in the absence of candidate agent;

(b) determining a level of ubiquitination of the Smad protein or variant thereof; and

(c) comparing the level of ubiquitination to a control level of ubiquitination in the absence of candidate agent, and therefrom determining whether the candidate agent modulates BMP-mediated signaling.

11. A method according to claim 10, wherein the method comprises a coupled ubiquitination assay.

12. A method according to claim 10, wherein the ubiquitinated HECT E3 ubiquitin ligase is present within a cell extract fraction.

13. A method according to claim 10, wherein the level of ubiquitination is determined by Western blot analysis.

14. A method according to claim 10, wherein the Smad protein or variant thereof comprises a tag.

15. A method for screening for an agent that modulates BMP-mediated signaling, comprising the steps of:

(a) contacting a cell that expresses a BMP receptor with a bone morphogenic protein and a candidate agent; and

(b) detecting a level of a Smad protein in the bone cell, relative to a level of the Smad protein in a cell that is contacted with the bone morphogenic protein in the absence of the candidate agent, and therefrom determining whether the candidate agent is a modulator of BMP-mediated signaling.

16. A method according to claim 15, wherein the Smad protein is Smad1 or Smad5.

17. A method according to claim 15, wherein the cell is a bone cell.

18. A method according to claim 15, wherein the cell is a neuron **[CORRECT CELL TYPE?]** or _____. **[OTHER IMPORTANT CELL TYPES?]**

19. A method according to claim 15, wherein the agent enhances BMP-mediated signaling.

20. A method for screening for an agent that modulates BMP-mediated signaling, comprising the steps of:

(a) contacting a cell that expresses a BMP receptor with a bone morphogenic protein and a candidate agent; and

(b) detecting a level of ubiquitination of a Smad protein in the cell, relative to a level of the Smad protein ubiquitination in a cell that is contacted with the bone morphogenic protein but is not contacted with the candidate agent, and therefrom determining whether the candidate agent modulates BMP-mediated signaling.

21. A method according to claim 20, wherein the Smad protein is Smad1 or Smad5.

22. A method according to claim 20 wherein the cell is a bone cell.

23. A method according to claim 20, wherein the cell is a neuron **[CORRECT CELL TYPE?]** or _____. **[OTHER IMPORTANT CELL TYPES?]**

24. A method according to claim 20, wherein the agent enhances BMP-mediated signaling.

25. A method for screening for an agent that modulates BMP-mediated signaling, comprising the steps of:

(a) contacting a cell that expresses a BMP receptor with bone morphogenic protein and a candidate agent; and

(b) detecting a level of a HECT E3 ubiquitin ligase activity in the cell, relative to a level of HECT E3 ubiquitin ligase activity in a cell that is contacted with the bone morphogenic protein in the absence of the candidate agent, and therefrom determining whether the candidate agent modulates BMP-mediated signaling.

26. A method according to claim 25, wherein the cell is a bone cell.

27. A method according to claim 25, wherein the cell is a neuron **[CORRECT CELL TYPE?]** or _____. **[OTHER IMPORTANT CELL TYPES?]**

28. A method according to claim 25, wherein the agent enhances BMP-mediated signaling.

29. A method for augmenting BMP-mediated signaling in a cell, comprising contacting a cell with an agent that inhibits binding of a HECT E3 ubiquitin ligase WW domain to a Smad PY motif.

30. A method for augmenting BMP-mediated signaling in a cell, comprising contacting a cell with an agent that inhibits ubiquitination of a Smad protein.

31. A method for stimulating bone formation in a patient, comprising administering to a patient a therapeutically effective amount of an agent that inhibits binding of a HECT E3 ubiquitin ligase WW domain to a Smad PY motif.

32. A method for stimulating bone formation in a patient, comprising administering to a patient a therapeutically effective amount of an agent that inhibits ubiquitination of a Smad protein.

33. A method for preventing or treating a condition associated with insufficient BMP-mediated cell signaling, comprising administering to a patient a

therapeutically effective amount of an agent that inhibits binding of a HECT E3 ubiquitin ligase WW domain to a Smad PY motif.

34. A method for preventing or treating a condition associated with insufficient BMP-mediated cell signaling, comprising administering to a patient a therapeutically effective amount of an agent that inhibits ubiquitination of a Smad protein.

35. A method according to claim 33 or claim 34, wherein the condition is selected from the group consisting of _____ **[LIST SOME?]**

METHODS FOR MODULATING BMP-MEDIATED SIGNALING

Abstract of the Disclosure

Methods are provided for identifying agents that modulate signaling mediated by bone morphogenic protein (BMP). Such agents may be identified using screens that evaluate candidate agents for the ability to modulate Smad protein degradation. Agents identified as described herein may be used to augment or inhibit BMP-mediated signaling in a variety of cell types and for therapeutic purposes.

FIG. 1

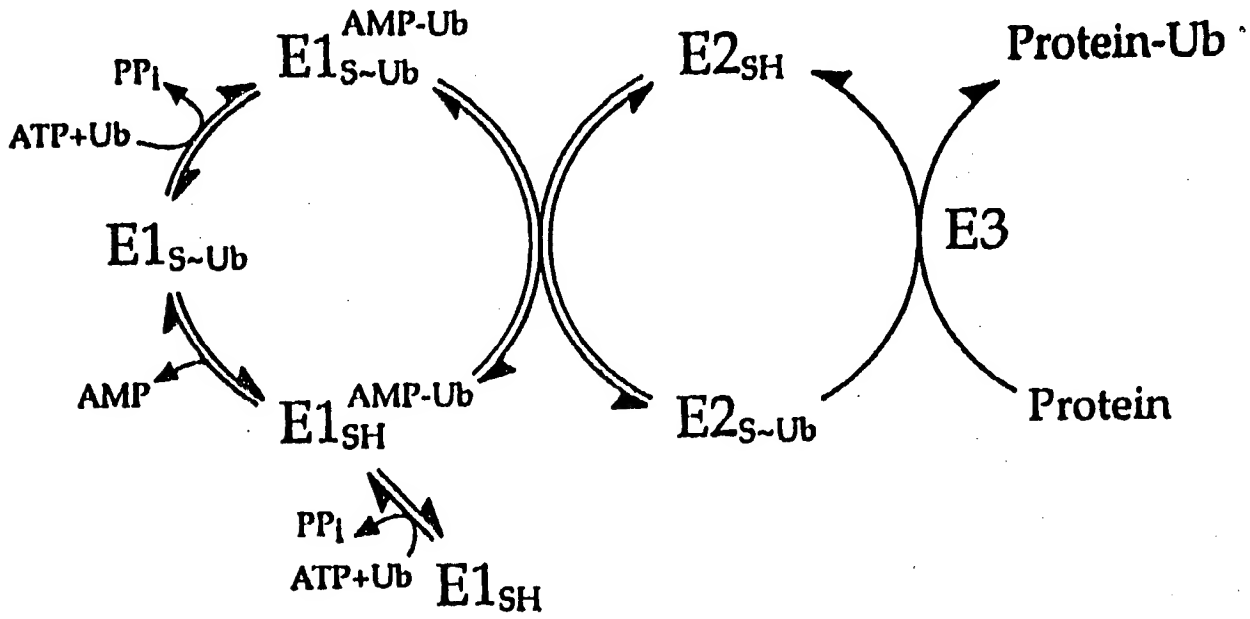

PPi
ATP+Ub
E1 S~Ub AMP-Ub
E1 S~Ub
AMP
E1 SH AMP-Ub
PPi
ATP+Ub
E1 SH
E2 SH
E2 S~Ub
E3
Protein-Ub
Protein

FIG. 2

BMP	–	–	+	+	+
LLF	–	+	–	+	+

FIG. 3

BMP	–	–	–	+	+
LLF	–	–	+	–	+
Ub	–	+	+	+	+

FIG. 4

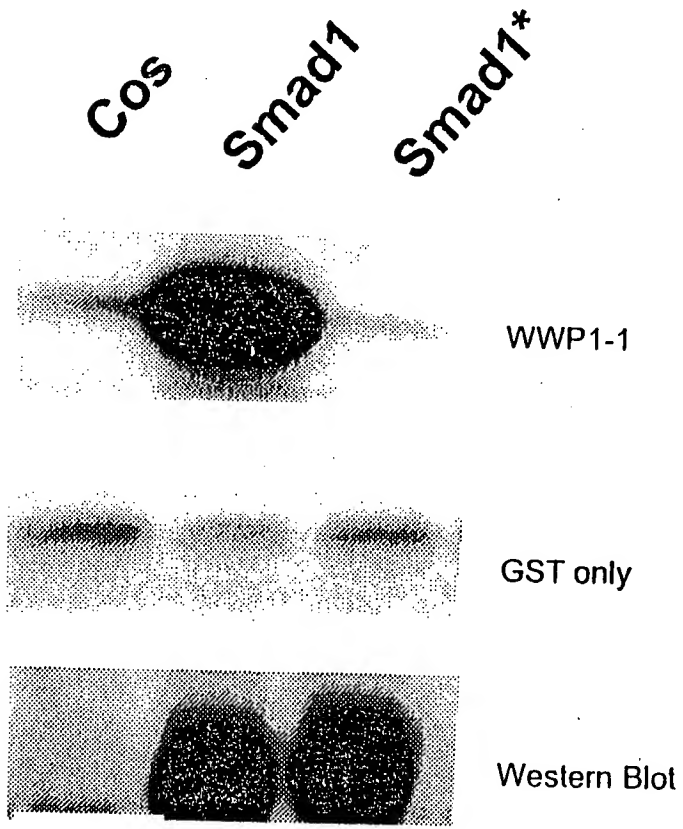


FIG. 5A-5D

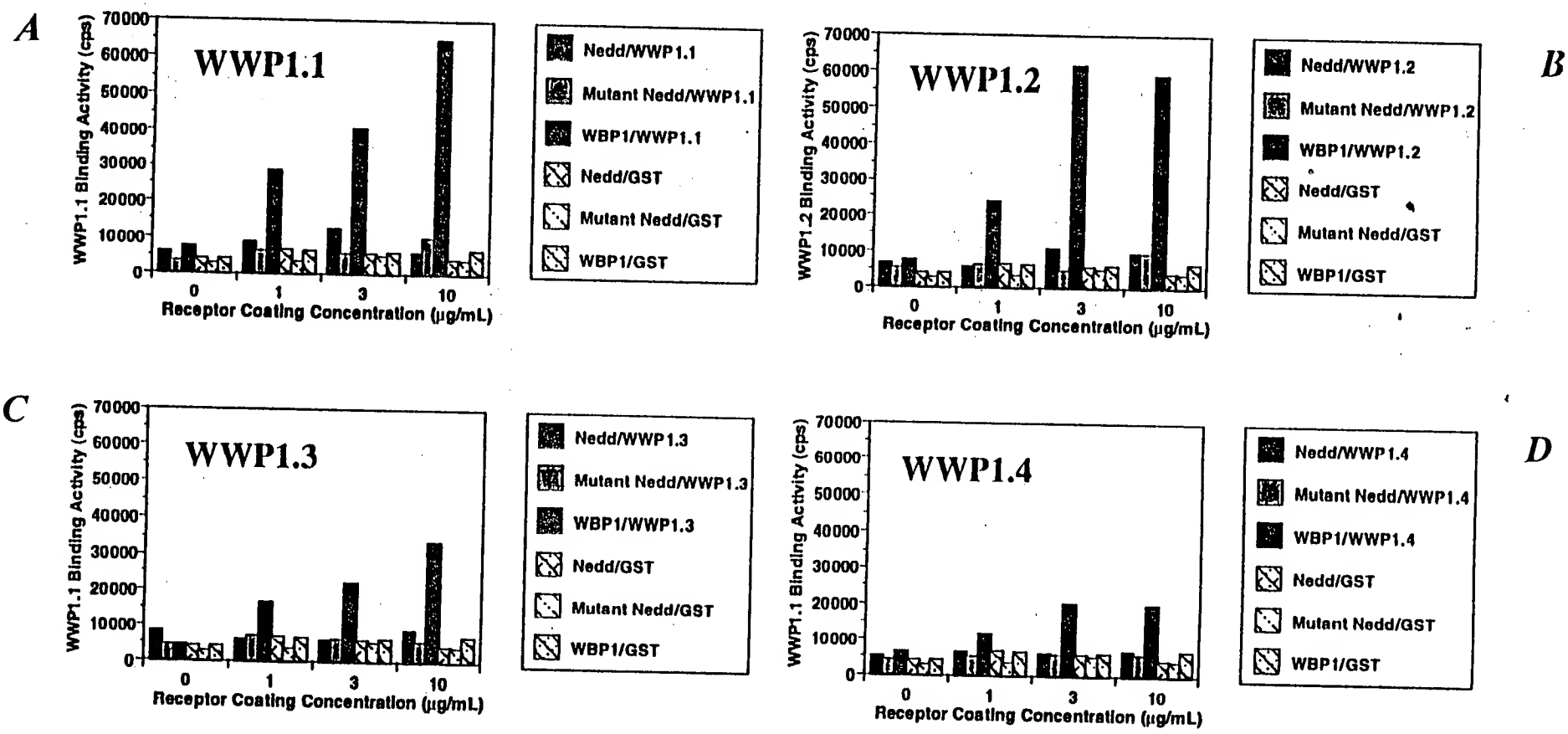

A
WWP1.1
WWP1.1 Binding Activity (cps)
70000
60000
50000
40000
30000
20000
10000
0
0
1
3
10
Receptor Coating Concentration (µg/mL)
Nedd/WWP1.1
Mutant Nedd/WWP1.1
WBP1/WWP1.1
Nedd/GST
Mutant Nedd/GST
WBP1/GST
B
WWP1.2
WWP1.2 Binding Activity (cps)
Receptor Coating Concentration (µg/mL)
Nedd/WWP1.2
Mutant Nedd/WWP1.2
WBP1/WWP1.2
Nedd/GST
Mutant Nedd/GST
WBP1/GST
C
WWP1.3
WWP1.1 Binding Activity (cps)
Receptor Coating Concentration (µg/mL)
Nedd/WWP1.3
Mutant Nedd/WWP1.3
WBP1/WWP1.3
Nedd/GST
Mutant Nedd/GST
WBP1/GST
D
WWP1.4
WWP1.1 Binding Activity (cps)
Receptor Coating Concentration (µg/mL)
Nedd/WWP1.4
Mutant Nedd/WWP1.4
WBP1/WWP1.4
Nedd/GST
Mutant Nedd/GST
WBP1/GST

FIG. 6A-6D

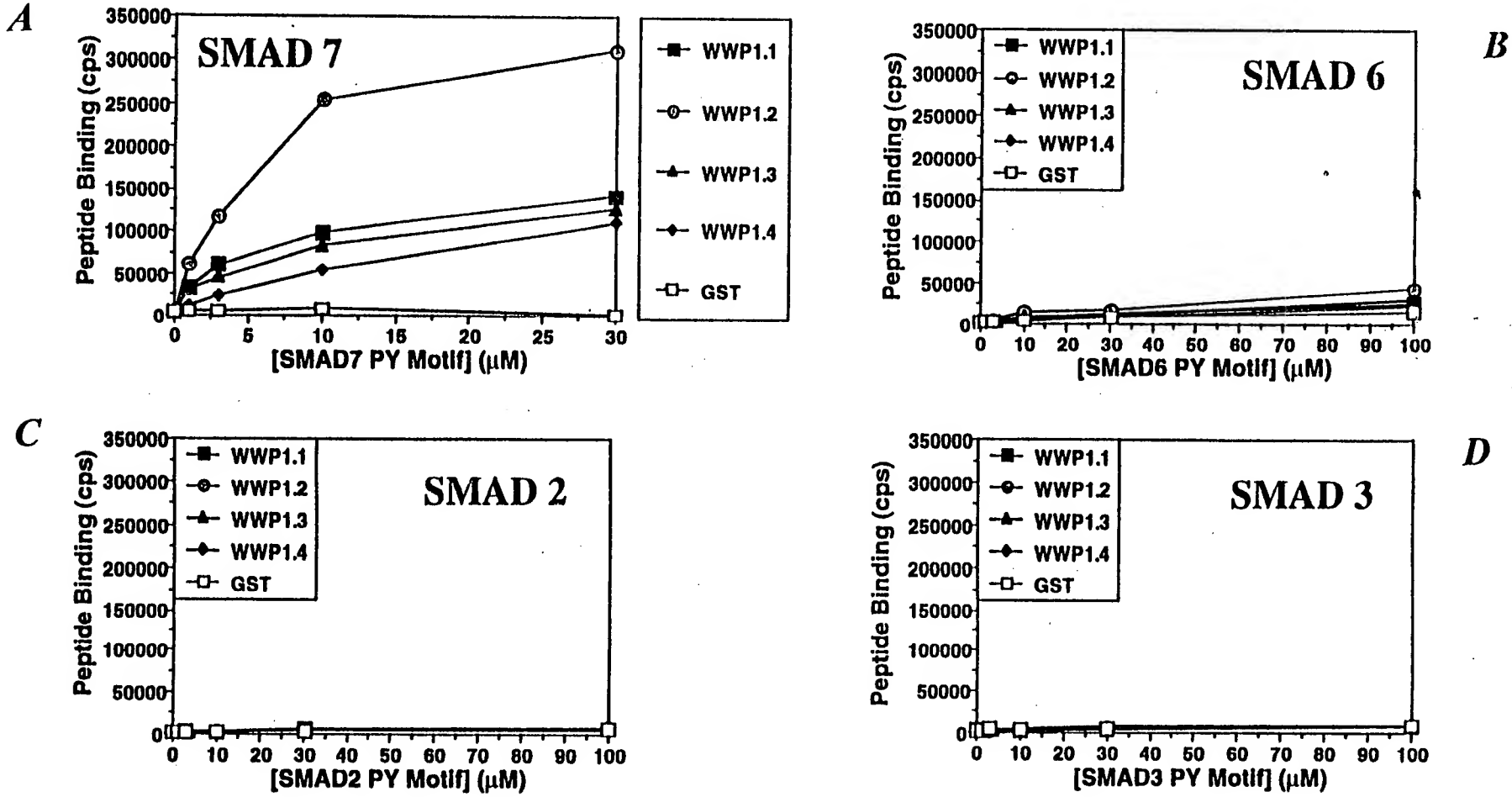
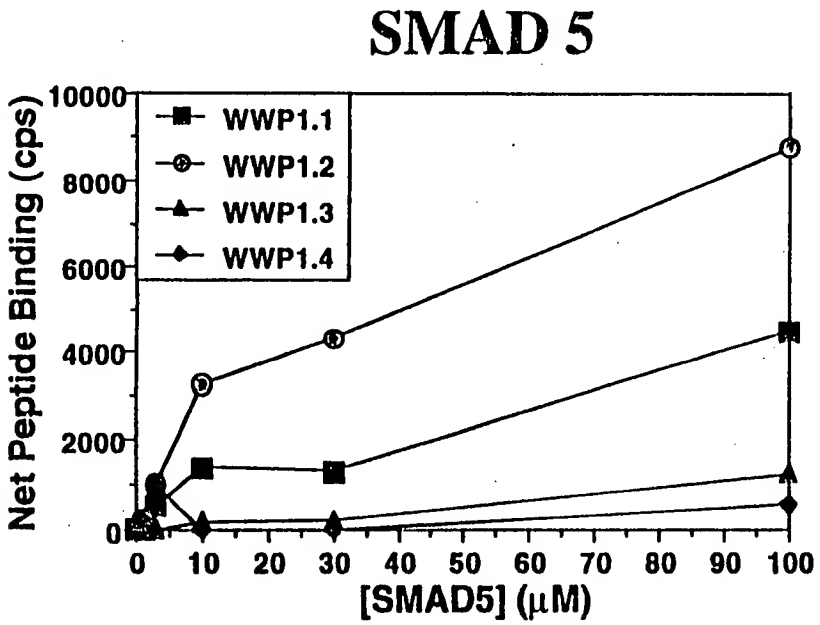

A
SMAD 7
Peptide Binding (cps)
350000
300000
250000
200000
150000
100000
50000
0
0
5
10
15
20
25
30
[SMAD7 PY Motif] (μM)
WWP1.1
WWP1.2
WWP1.3
WWP1.4
GST
B
SMAD 6
Peptide Binding (cps)
0
10
20
30
40
50
60
70
80
90
100
[SMAD6 PY Motif] (μM)
C
SMAD 2
[SMAD2 PY Motif] (μM)
D
SMAD 3
[SMAD3 PY Motif] (μM)

FIG. 7A and 7B

A



B

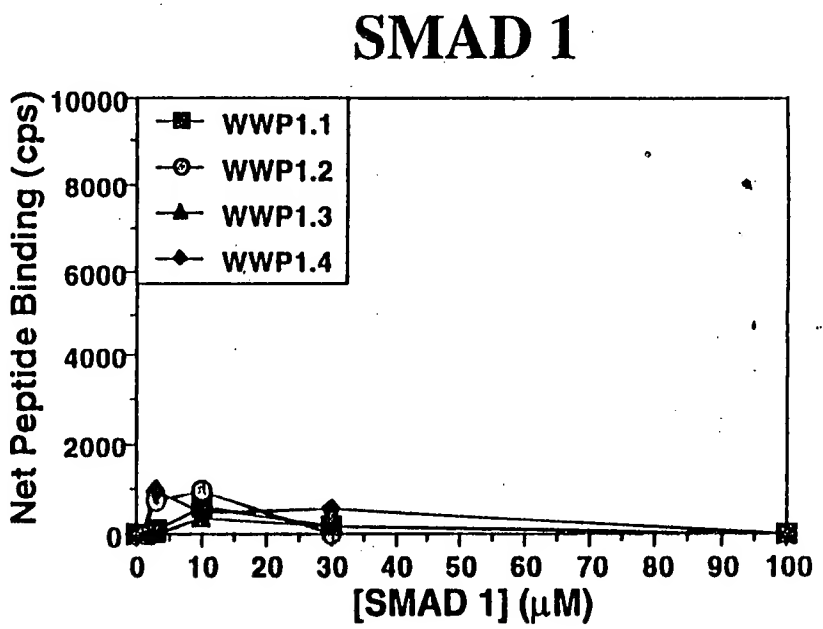


FIG. 8A and 8B

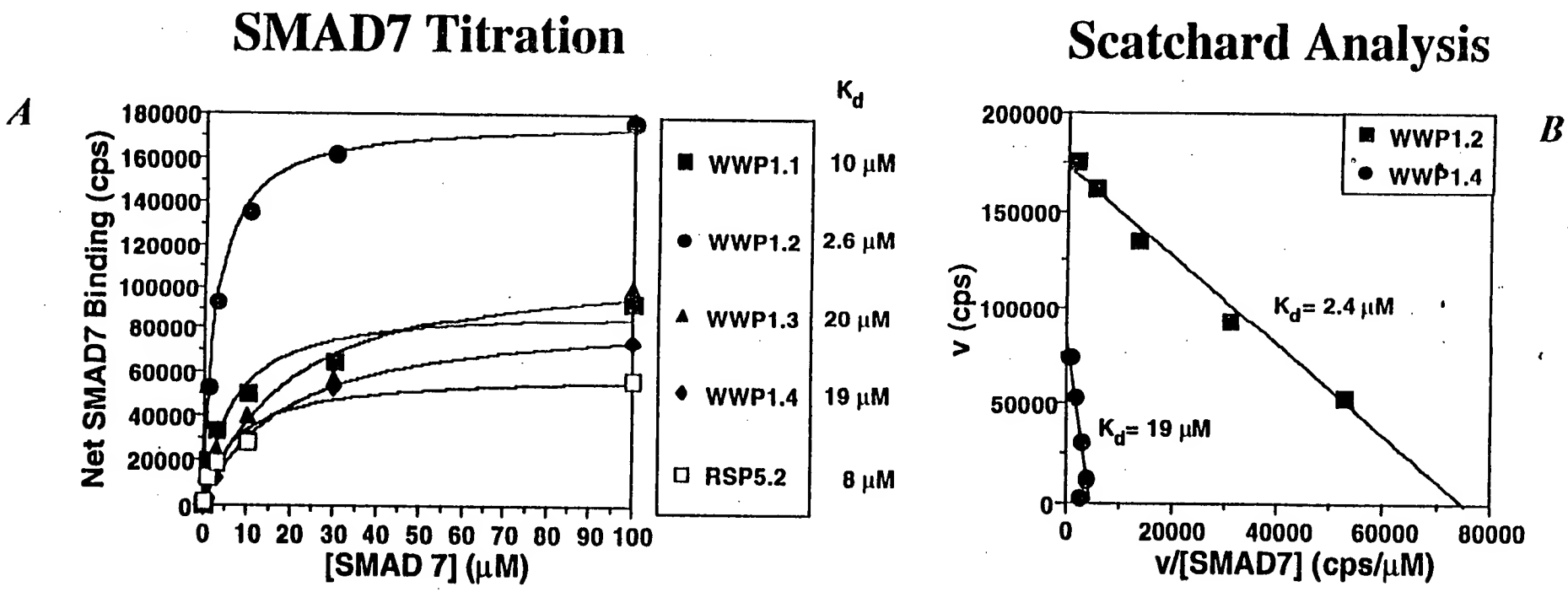

SMAD7 Titration
A
Net SMAD7 Binding (cps)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0 10 20 30 40 50 60 70 80 90 100
[SMAD 7] (μM)
Kd
WWP1.1 10 μM
WWP1.2 2.6 μM
WWP1.3 20 μM
WWP1.4 19 μM
RSP5.2 8 μM
Scatchard Analysis
B
v (cps)
200000
150000
100000
50000
0
0 20000 40000 60000 80000
v/[SMAD7] (cps/μM)
WWP1.2
WWP1.4
Kd= 2.4 μM
Kd= 19 μM

FIG. 9A-9C

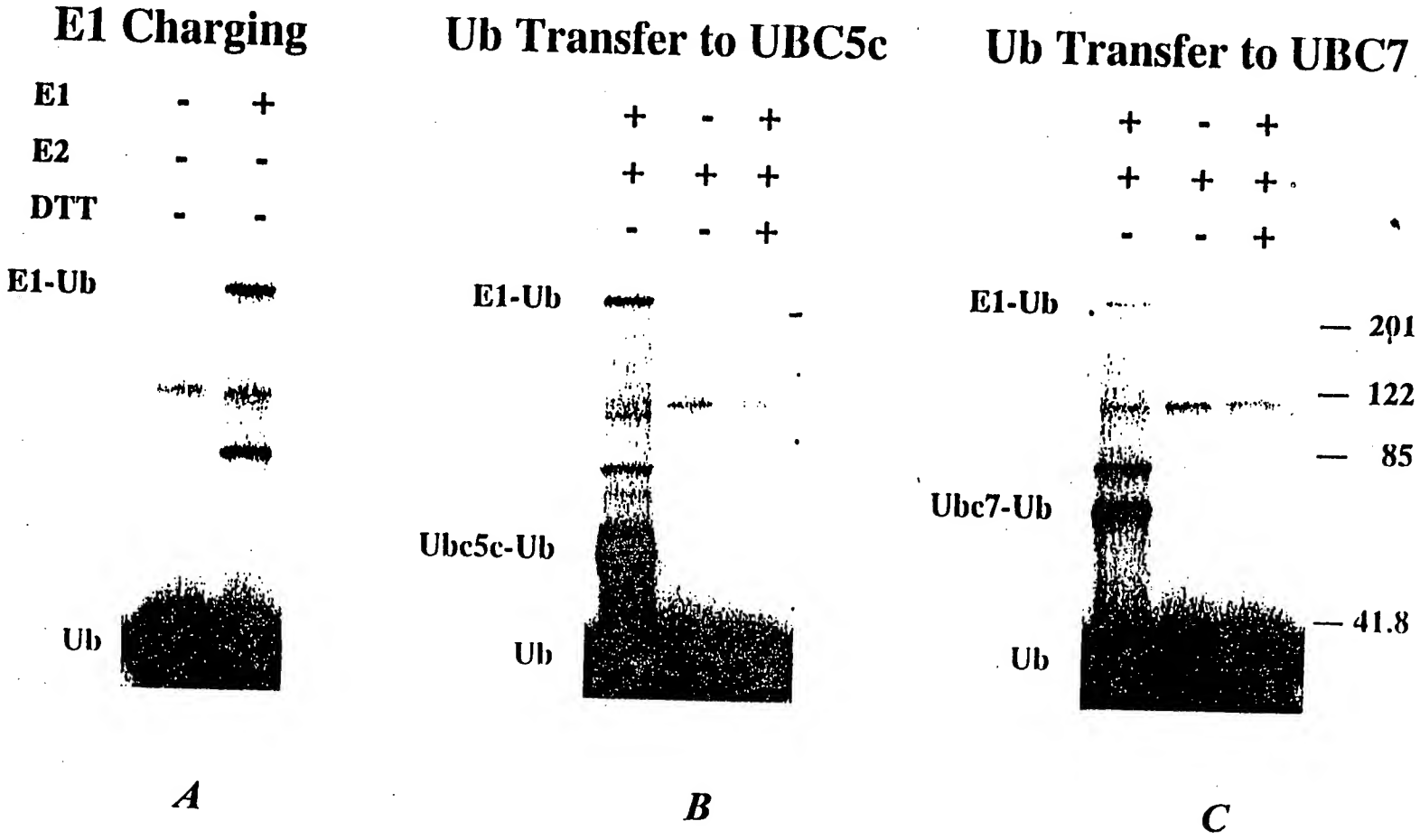

E1 Charging
E1 - +
E2 - -
DTT - -
E1-Ub
Ub
A
Ub Transfer to UBC5c
+ - +
+ + +
- - +
E1-Ub
Ubc5c-Ub
Ub
B
Ub Transfer to UBC7
+ - +
+ + +
- - +
E1-Ub
— 201
— 122
— 85
Ubc7-Ub
— 41.8
Ub
C

FIG. 10A-10C

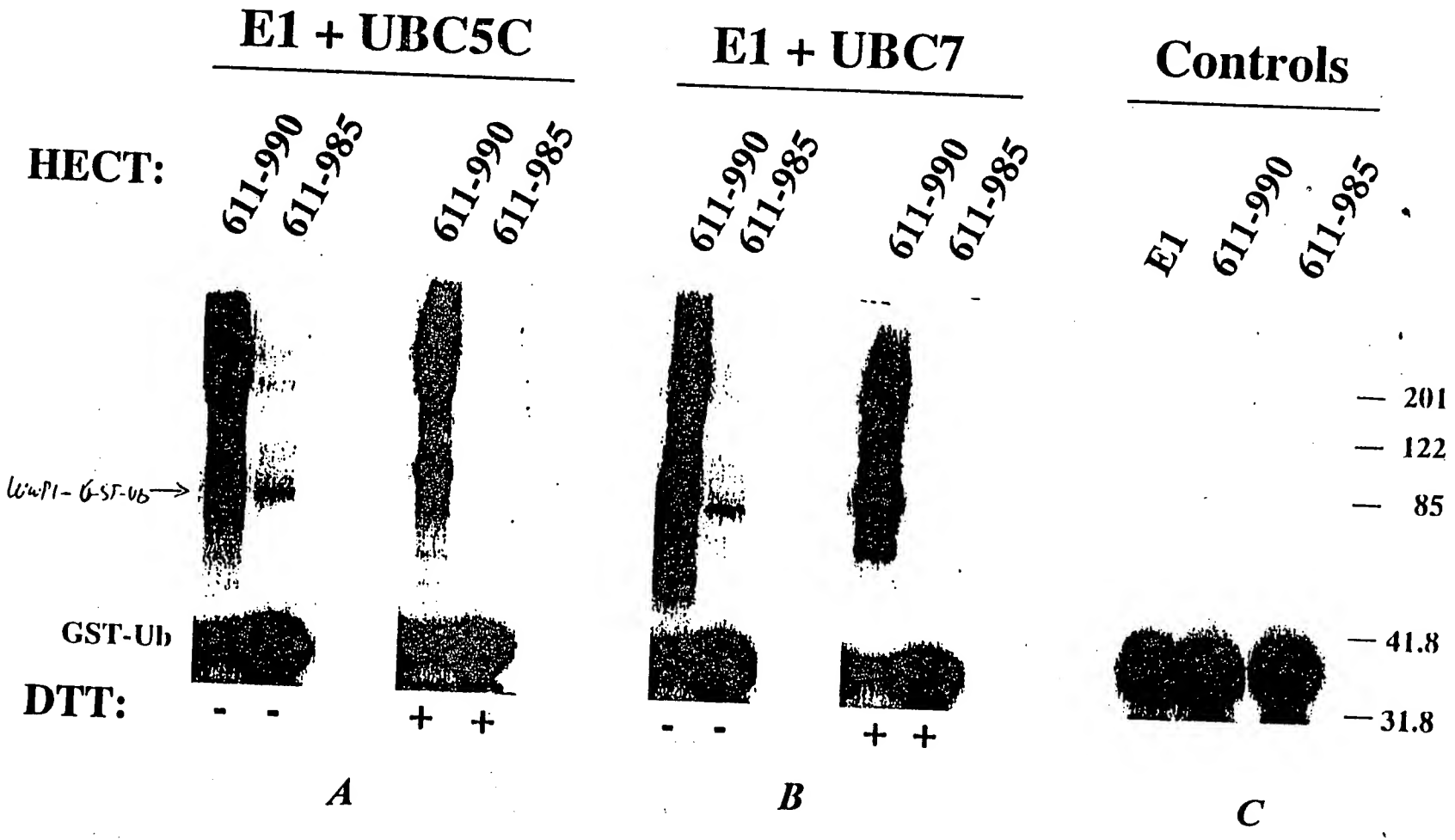

E1 + UBC5C
E1 + UBC7
Controls
HECT:
611-990
611-985
611-990
611-985
611-990
611-985
611-990
611-985
E1
611-990
611-985
Wwp1-GST-Ub
GST-Ub
DTT:
- -
+ +
- -
+ +
201
122
85
41.8
31.8
A
B
C

FIG. 11A-11C

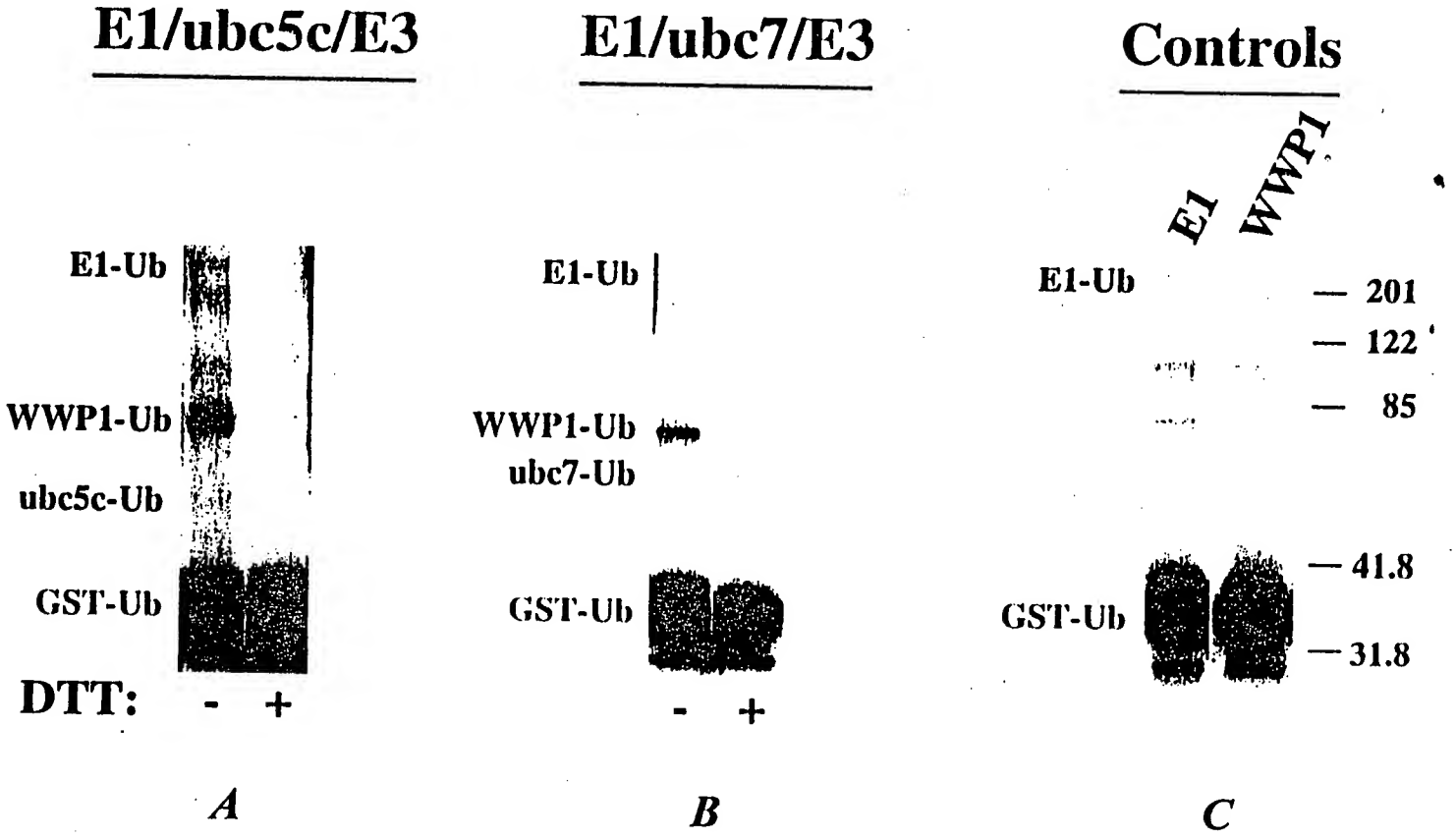
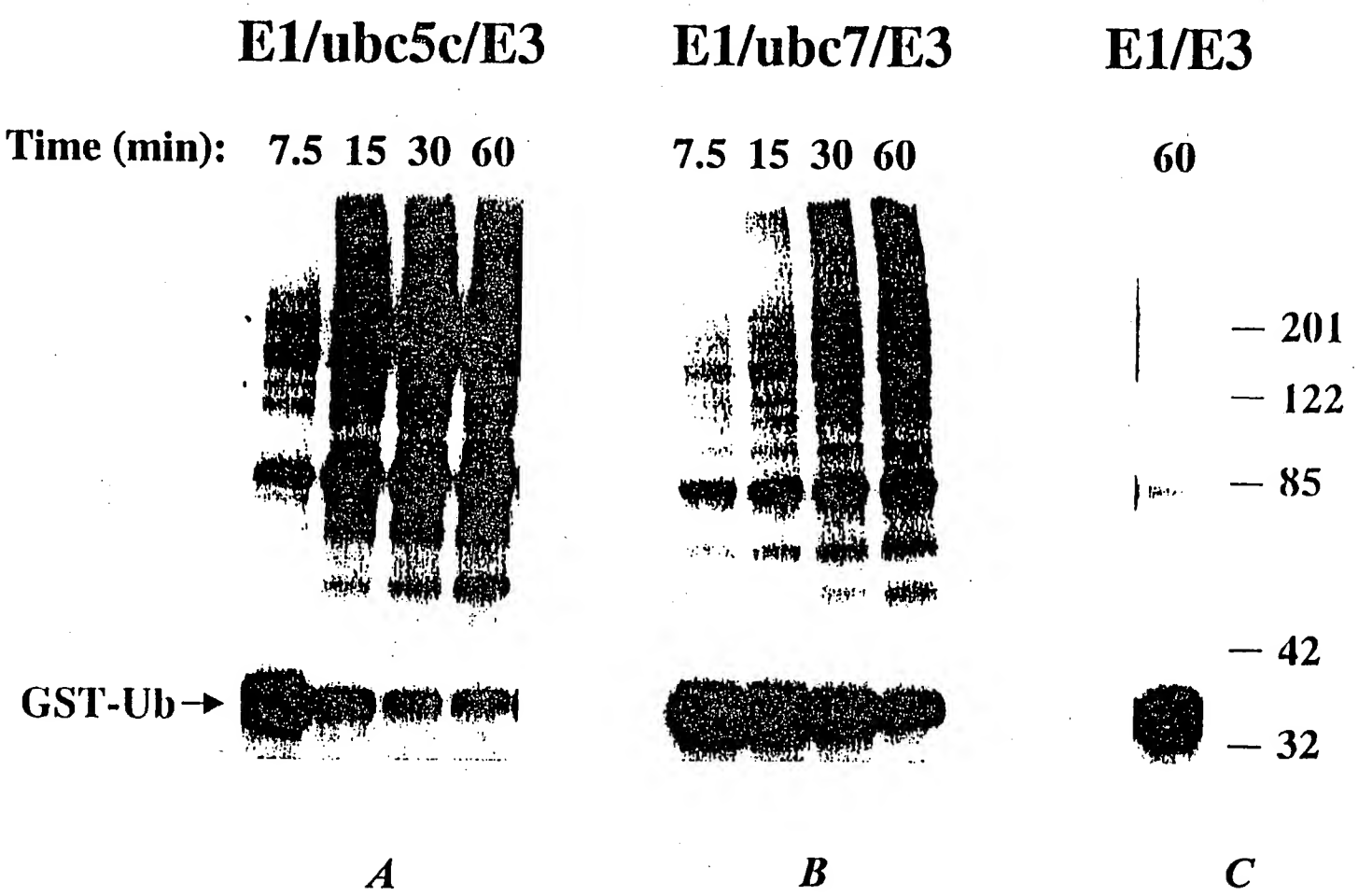

E1/ubc5c/E3
E1-Ub
WWP1-Ub
ubc5c-Ub
GST-Ub
DTT: - +
A
E1/ubc7/E3
E1-Ub
WWP1-Ub
ubc7-Ub
GST-Ub
- +
B
Controls
E1
WWP1
E1-Ub
201
122
85
GST-Ub
41.8
31.8
C

FIG. 12A-12C


E1/ubc5c/E3
E1/ubc7/E3
E1/E3
Time (min):
7.5 15 30 60
7.5 15 30 60
60
GST-Ub
201
122
85
42
32
A
B
C